Form Approved Through REDACTED
OMB No. 0925-0001

AA

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PROGRAM PUBLIC HEALTH SERVICE
PROJECT **GRANT APPLICATION**
Follow instructions carefully. Type in the unshaded areas only. Type density must be 10 c.p.i.

LEAVE BLANK FOR PHS USE ONLY.		
Type	Activity	Number
Review Group		Formerly
Council/Board *(Month, Year)*		Date Received

1. TITLE OF PROJECT (Do not exceed 56 typewriter spaces.)
NEW METHODS FOR CANCER DETECTION

2a. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT ☒ NO ☐ YES *(If "YES," state number and title)*
Number: Title: CONFIDENTIAL INFORMATION

2b. TYPE OF GRANT PROGRAM P01

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR

3a. NAME *(Last, first, middle)*
BARANY, FRANCIS

3b. DEGREE(S)
PH.D.

3c. SOCIAL SECURITY NO.
REDACTED

3d. POSITION TITLE
ASSOCIATE PROFESSOR

3e. MAILING ADDRESS *(Street, city, state, zip code)*
CORNELL UNIV. MEDICAL COLLEGE
1300 YORK AVENUE
NEW YORK, NY 10021

3f. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
MICROBIOLOGY

3g. MAJOR SUBDIVISION
CORNELL UNIV. MEDICAL COLLEGE

3h. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: 212 746-6509
FAX: 212 746-8587

BITNET/INTERNET ADDRESS BARANY@CUMC.CORNELL.EDU

4. HUMAN SUBJECTS — If "Yes," exemption no. or IRB approval date — 4b. Assurance of compliance no.
☒ 4a. NO ☐ YES

5. VERTEBRATE ANIMALS — If "Yes," IACUC approval date — 5b. Animal welfare assurance no.
☒ 5a. NO ☐ YES

6. DATES OF ENTIRE PROPOSED PROJECT PERIOD
From (MMDDYY): REDACTED
Through (MMDDYY): REDACTED

7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD
7a. Direct Costs ($): $971,041
7b. Total Costs ($): $1,224,862

8. COSTS REQUESTED FOR ENTIRE PROPOSED PROJECT PERIOD
8a. Direct Costs ($): $5,295,869
8b. Total Costs ($): $6,995,293

9. PERFORMANCE SITES *(Organizations and addresses)*
CORNELL UNIV. MEDICAL COLLEGE
1300 YORK AVENUE
NEW YORK, NY 10021

10. INVENTIONS AND PATENTS *(Competing continuation application only)*
☐ NO ☐ YES If "YES," ☐ Previously reported ☐ Not previously reported

11. NAME OF APPLICANT ORGANIZATION
CORNELL UNIVERSITY MEDICAL COLLEGE
ADDRESS 1300 YORK AVENUE
NEW YORK, NY 10021

12. TYPE OF ORGANIZATION
☐ Public: *Specify* ☐ *Federal* ☐ *State* ☐ *Local*
☒ Private Nonprofit
☐ Forprofit *(General)* ☐ Forprofit *(Small Business)*

13. ENTITY IDENTIFICATION NUMBER
1131623978A1

Congressional District
14

14. BIOMEDICAL RESEARCH SUPPORT GRANT CREDIT
Code: 01 Identification: SCHOOL OF MEDICINE

15. NAME OF ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
PHILIP V. GIUCA
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FAX 212 746-8745
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NEW YORK, NY 10021

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16. NAME OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
GREGORY W. SISKIND
TELEPHONE 212 746-6020
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NEW YORK, NY 10021

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17. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Willful provision of false information is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me to civil penalties under the Program Fraud Civil Remedies Act of REDACTED (45 CFR 79).

SIGNATURE OF PERSON NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*
Dr. F. Barany

DATE
REDACTED

18. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense (U.S. Code, Title 18, Section 1001). I am aware that any false, fictitious, or fraudulent statement may, in addition to other remedies available to the Government, subject me

SIGNATURE OF PERSON NAMED IN 16. *(In ink. "Per" signature not acceptable.)*

DATE
REDACTED

REDACTED

(ii) Development of a multiplex ligase detection reaction/polymerase chain reaction (LDR/PCR) system for detection of gene amplifications and deletions in tumor biopsies. This study aims to develop a technology to investigate the amplification and deletion of several genes in a single assay. We have designed ligase detection primers for the following genes: SOD (on chromosome 21q), G6PD (on chromosome Xq), p53 tumor suppressor gene (on chromosome 17p), HER-2/neu (on chromosome 17q), and Int-2 (on chromosome 11q). Each set of LDR primers includes the same external "zip code" sequences, which will allow for proportional amplification of all five chromosome probes with a single pair of PCR primers. The method will be validated with genomic DNA samples from normal, and trisomy 21 human males and females. Subsequently, cell lines with known HER-2/neu and int-2 gene amplifications, and p53 deletions will be used to test the sensitivity of this technique. The assay will be extended to frozen and fixed breast tumor material. Initial studies will investigate the 100 to 200 tumors to determine the feasibility of large scale studies correlating multiple genetic alterations to the clinical/biological behavior of breast cancers.

We will use the Ligase Detection Reaction (LDR) in the first step of gene quantification to create artificial PCR templates (see Fig. 15). The LDR products will be in the same ratio as the genes from which they were derived, but will also have the same artificial PCR primer sequences (which we call "zip code" sequences) at their ends. Using only two PCR zip code primers, all of the LDR templates will be amplified together, maintaining proportionality. We will fluorescently label one of the zip code primers to allow quantification on a DNA sequencing apparatus. Because the LDR templates will include a restriction enzyme site at a unique position, restriction digestion of the PCR products will permit us to distinguish the products from different genes on the basis of their lengths. We will first test the technology by identifying the dosages of genes on chromosomes X and 21 in male, female and trisomy 21 DNA's. Investigations of deletion of p53, and amplification of HER-/neu and int-2 in breast tumors and cell lines will follow.

(a) Design, synthesis and test of LDR/PCR oligonucleotides using known DNA samples. Oligonucleotides will be synthesized to recognize exons in SOD (on chromosome 21q), G6PD (on chromosome Xq), p53 tumor suppressor gene (on chromosome 17p), HER-2/neu (on chromosome 17q), and Int-2 (on chromosome 11q). These oligonucleotide primers have been designed so their ligation products have unique internal sequences, but the overall length and G+C content are identical (see Fig. 16). To minimize differences in ligation rates, the exon specific region of each primer set was chosen to ligate the junction sequence of (A,T)C↓C(A,T). This junction sequence corresponds to either a proline residue (codon CCN) or on the noncoding strand opposite a tryptophan residue (TGG). This particular sequence was chosen since tryptophan and proline residues tend to be conserved, and less likely to be a site of polymorphism. In addition, each sequence contains a single *Hae*III or *Hin*P1I restriction site at slightly different positions. Adjacent to the internal gene specific sequences are "adjustment sequences" (white bars), which equalize product length at 96 bases and G+C content at 52%. The external sequences of all oligonucleotides will be the same and complimentary to a pair of "zip code" PCR primers that will amplify only ligated products and not human sequences. One of the zip code PCR primers will be fluorescently labeled, while the other will contain a biotin group.

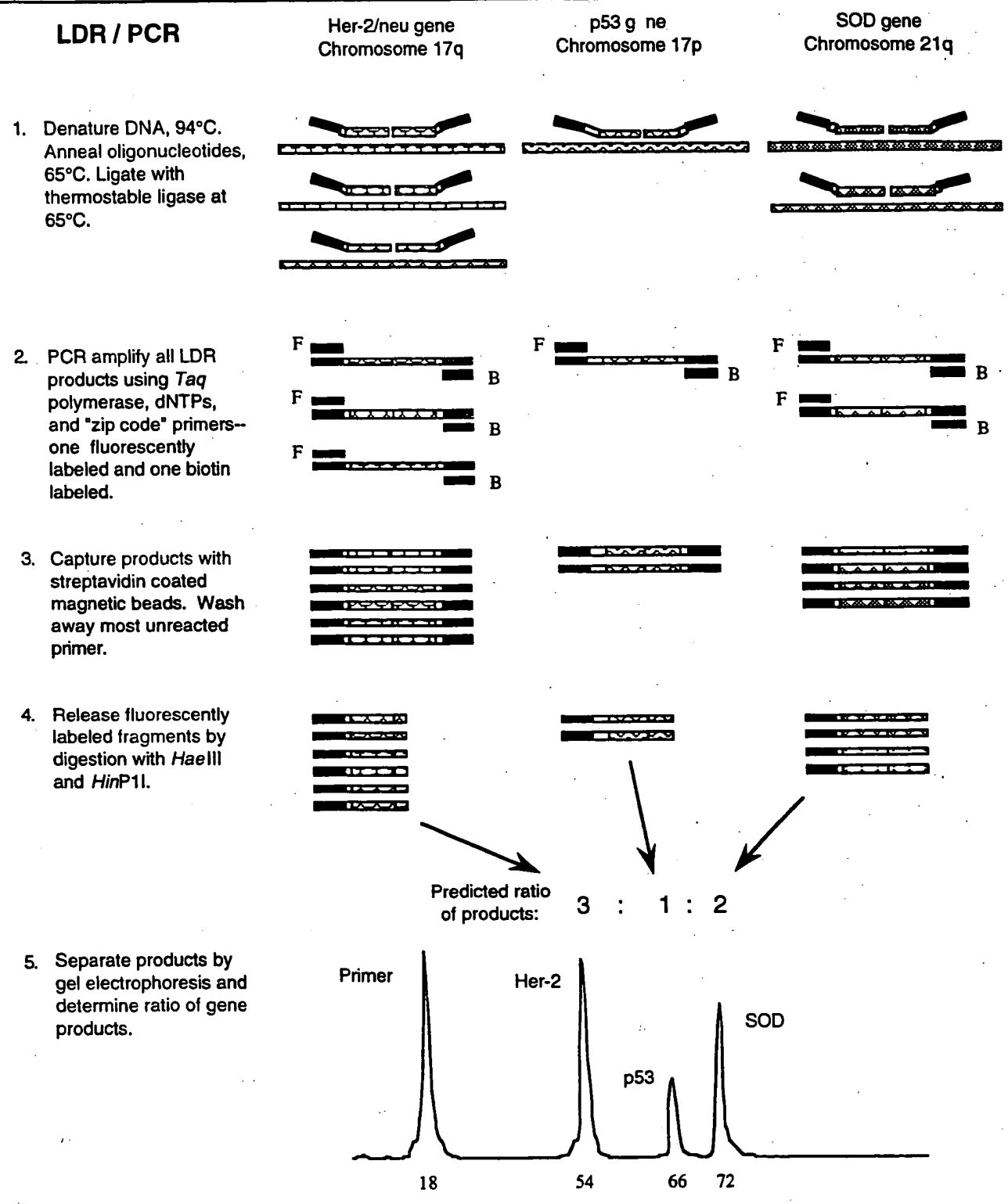

Fig. 15. Quantification of gene amplifications and deletions using the ligase detection reaction coupled to the polymerase chain reaction (LDR/PCR). Following denaturation, pairs of LDR primers anneal to their complementary templates and are ligated. Conditions will be optimized to obtain equal ligation efficiencies for all primer sets. Ligation with *Tth* ligase will be performed at 65°C well below the primers' designed T_m values of 75°C. All LDR products will be amplified simultaneously with *Taq* polymerase using two common "zip code" primers, thus maintaining proportionality. One zip code primer contains a fluorescent group, and the other a biotin group. Products may then be captured with streptavidin coated magnetic beads, washed, and digested with *Hae*III and *Hin*P1I to release fluorescently labeled fragments of unique size. These products may be separated on an ABI 373A DNA sequencer, and their ratio will be used to determine the relative copies of genes present in the initial target sample.

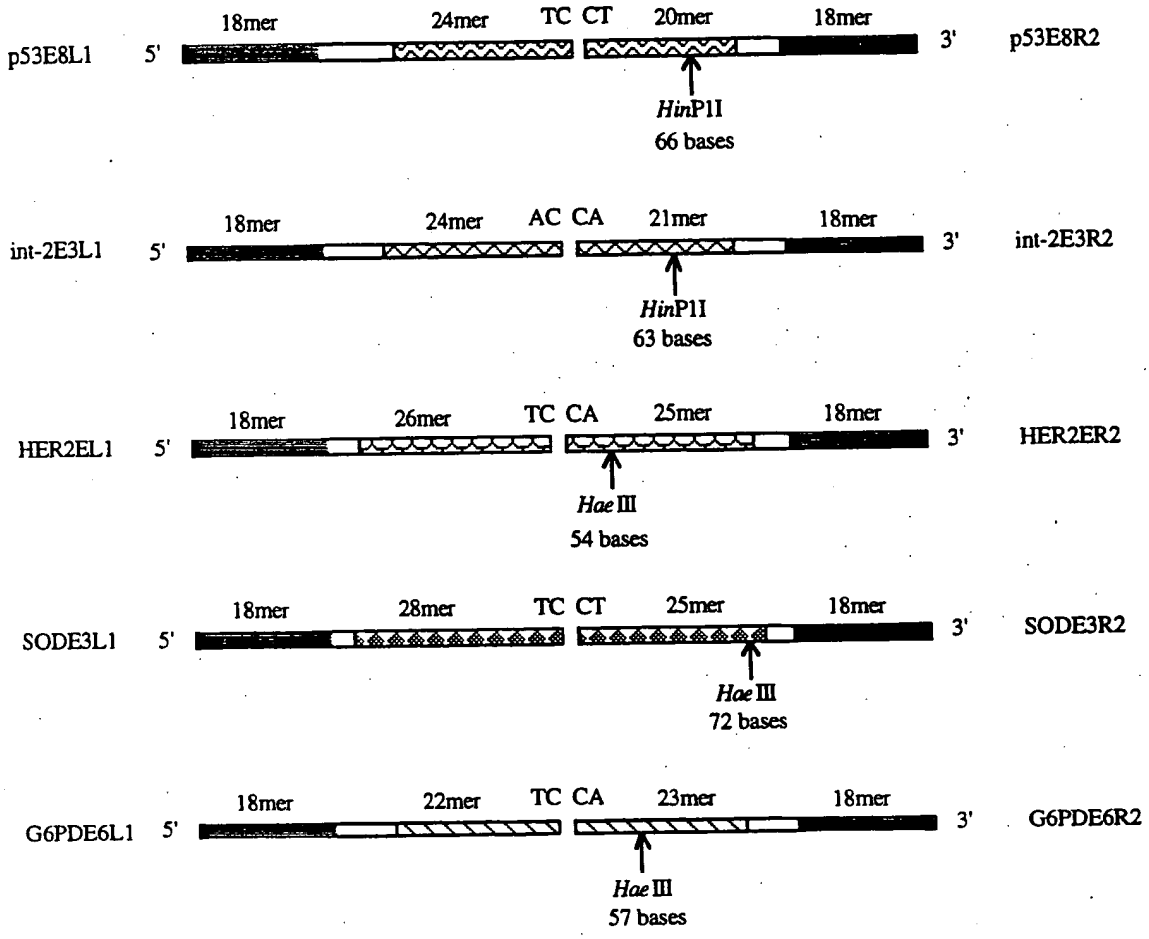

Fig. 16. LDR primers for quantification of gene amplifications and deletions. Oligonucleotides have been designed to recognize exon 8 in the p53 tumor suppressor gene (on chromosome 17p), exon 3 of int-2 (on chromosome 11q), an internal exon in HER-2/neu (on chromosome 17q), exon 3 in SOD (on chromosome 21q), and exon 6 in G6PD (on chromosome Xq). Each LDR primer pair has the following features: (i) The left primer contains from 5' to 3' an 18 base sequence identical to the fluorescently labeled zip code primer (black bar), an "adjustment sequence" (white bar), and a gene-specific sequence of from 22 to 28 bases with a T_m of 75°C (patterned bar). (ii) The right primer contains from 5' to 3' a gene-specific sequence of 20 to 25 bases with a T_m of 75°C (patterned bar), a single *Hae*III or *Hin*P1I restriction site at slightly different positions within the gene-specific sequence, an "adjustment sequence" (white bar), and an 18 base sequence complementary to the biotinylated zip code primer (black bar). (iii) The adjustment sequences (white bars) are designed such that the combined length of the two primers is exactly 96 bases, with 50 G+C bases and 46 A + T bases. The position of each unique restriction site generates a product which differs by at least 2 bases from the other products. (iv) The exon specific region of each primer set was chosen to ligate the junction sequence of (A,T)C↓C(A,T). This junction sequence corresponds to either a proline residue (codon CCN) or the complementary sequence of a tryptophan residue (TGG). These sequences were chosen to minimize differences in ligation rates and chance of a polymorphism at the ligation junction. Sequences of oligonucleotides are available upon request.

Ligation reactions will be carried out using test genomic DNA and the appropriate oligonucleotides (e.g. SOD and G6PD oligonucleotides for male, female, and trisomy 21 DNA's). Several ligation conditions will be tested to assure equal rates of ligation for each primer set. Ideally, ligations should go to completion, so initial ligations tested will be 10°C below the oligonucleotide T_m value. The amount of ligation product may be proportionally increased by a few additional LDR cycles (1 to 5 cycles), but not so many as to have product inhibition of the ligation reaction. After ligation, *Taq* polymerase with the zip code primers will be added, and PCR amplification will be performed. (We are aware that one LDR oligonucleotide may also serve as a primer and the other as a template for polymerase extension. Although products from those reactions may hybridize to generate a substrate for further PCR amplification, such products will not form in the absence of genomic DNA, and will be an insignificant percentage of the authentic LDR/PCR product.)

Products will be captured with streptavidin coated magnetic beads, washed of unreacted fluorescent primers, and released by digestion with *Hae*III and *Hin*P1I. This will yield fluorescently labeled fragments of different sizes which can be separated and quantified on an ABI 373A DNA sequencer. The ratios of products will be determined using the Genescan 672 software. Multiple reactions will allow us to calculate means and 95% confidence intervals. As controls, different ratios of individual PCR amplified products will be mixed and diluted for LDR/PCR. To test for equal PCR efficiencies among the LDR products, a solution will be prepared with the LDR products in known ratios. By PCR amplifying from this solution and quantifying the products at different cycles we will be able to see whether the product ratios remain the same. It may be necessary to take samples after fewer rounds of PCR amplification to avoid skewing of the product ratios as the PCR reaction plateaus.

The above protocol may be generalized to accommodate quantification of gene amplification or deletion at several dozen loci simultaneously, without the need for a conveniently placed restriction site. Following LDR ligation, the products are proportionally amplified using one unlabeled and one biotinylated zip code primer. Products will be captured with streptavidin coated magnetic beads, washed of unreacted primers, and the DNA made single stranded by washing the beads in base. Addition of a molar excess of fluorescent zip code primer and several gene specific adjacent primers (containing different size tails) which hybridize on the single stranded product allows for quantitative (90% or better) ligation in the presence of thermostable ligase. Unreacted primers are removed by washing, and the ligated products released in a small volume of base. The fluorescently labeled ligation products will be of different sizes which can be separated and quantified on an ABI 373A DNA sequencer. Alternatively, the gene specific adjacent primers will contain array specific zip codes, which will be captured by the appropriate oligonucleotide or PNA addressable array, and quantified on a Molecular Dynamics Fluorimager 575 (See Project 5 and Core B.)

The ability to simultaneously detect both small and large deletions has additional benefits for cancer research. By judiciously selecting genes from both the long and short arms of each chromosome, this LDR/PCR method could be used to generate a molecular karyotype. This could help identify additional loci associated with tumorogenesis. Once a broad chromosomal region is identified, the same LDR/PCR method could help develop a fine structure map of the region, analogous to the initial discovery of DCC [101].

In addition, small deletions or insertions of unpredictable size in tumor suppressor genes might be easily identified by synthesizing sets of LDR primers to cover every 6-10 bases in the coding region of the gene. (This will become more feasible as improvements in oligonucleotide synthesis instrumentation will allow for synthesis of dozens to hundreds of oligonucleotides at a time.) For example, in a literature survey, we identified 151 mutations in the APC gene of which 15 were insertions (9.9%), 52 were deletions (34.4%) and 84 were single base changes. All 67 insertions and deletions (44.3%) could be easily identified by using just 25 sets of LDR primers, and one set of zip code primers. The remaining single base changes could be identified by the standard PCR/LDR methods described in specific aim *(i)* above.

(b) Sensitivity of quantification in mixed tumor/normal cell populations. The detection of X chromosome gene dosage in male and female genomic DNA's is a model for the deletion of a single allele in cancer cells. The additional chromosome in trisomy 21 genomic DNA mimics a 50 percent gene amplification. The sensitivity of our technology can be determined by examining these model situations and by mixing male and female and trisomic and normal cells. Quantification of LDR/PCR products from multiple reaction tubes will allow us to determine gene dosage with 95% confidence limits based on the *t* test. We will need to determine the number of observations (samples) required to achieve the appropriate confidence limits for detecting a single allele deletion or 50 percent gene amplification in half of the cells in a tumor. Initial experiments will use male, female, normal, and trisomic cells as well as mixtures of these DNA's (e.g. trisomic DNA diluted with normal DNA) to test a wide range of possible gene ratios. These studies will be extended to detect gene amplifications and deletions in previously characterized breast tumor cell lines. Dilution of cultured breast tumor cells with normal cells will also help us establish the limits of the LDR/PCR method.

(c) Detection of gene amplification or deletion in frozen and fixed tissue. Our sample set of 100 to 200 breast tumors will be investigated for deletion of p53, and amplification of HER-2/neu and/or int-2, compared to control genes SOD and G6PD by simultaneously amplifying LDR products from all these sites. Fixed

tumor specimens corresponding to the frozen samples will be examined to develop the technology for use on formaldehyde-treated, paraffin-embedded specimens. Bone marrow aspirate samples corresponding to these primary tumors have been investigated for micrometastases by immunohistochemical techniques. Many of them have also been characterized for HER-2/neu amplification by southern blotting [125]. The results of our studies of HER-2/neu and int-2 amplification in this tumor set will be correlated with the previously explored laboratory parameters and clinical prognostic parameters (e.g. primary tumor size, lymph node status and estrogen receptor status). We recognize that our p53 LDR primers have their junction in exon 8 at codon 278, and thus may fail to ligate when used on tumors containing a mutation at or near this codon. Such a result would be falsely interpreted as a p53 deletion instead of just a single base mutation. However, the interpretation of this data, namely, that the sample had lost a *functional* p53 gene remains the same.

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